Muntingia calabura Leaves Extract, a Potential Gastroprotective Agent against Gastric Mucosal Damage Induced by Soft Drink and Alcoholic Beverages

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ABSTRACT

Introduction. Exposures of alcoholic beverages and soft drinks have been notorious for their deleterious effect on gastric mucosal cell, causing disturbances on gastric mucosa. The high content of antioxidant in *Muntingia calabura* leaves have potentials to counteract the degeneration of gastric mucosal cells due to exposure of both drinks. This study aimed to evaluate the effects of flavonoids in *Muntingia calabura* ethanolic leaves extract (MCELE) as gastroprotective agents against the alcoholic beverages and soft drinks induced gastric mucosal damage. **Method.** Twenty four male wistar rats were divided into four groups respectively treated with 1,8 ml/200grBW 40%-alcoholic beverages (K1), 50 ml/day soft drink (K2), pre-treated with 500 mg/kg MCELE one hour before oral administration of 40%-alcoholic drinks (P1) and soft drink (P2). All rats were treated for 30 days. On the 31st day, the rats were teminated and the histological degrees of gastric mucosal damage were determined by modified scale of epithelial mucosa integrity *Barthel-Manja*. **Results.** The K1 and K2 group exhibited severe gastric mucosal injury, with observed ulceration percentages of 33,3% and 23,3% respectively. Meanwhile, the pre-treated MCELE groups (P1 and P2) exhibited significant protection of gastric mucosa histologically ($p<0.001$), showing 0,0% of ulceration on both groups. **Conclusion.** Soft drinks have degenerating effect as strong as the alcoholic beverages. The treatment with MCELE prior to 40%-alcoholic beverages and soft drinks has significantly protected gastric mucosa as ascertained by significant reduction of gastric mucosal injury and increase in normal gastric mucosa.

Keywords: alcohol, soft drinks, gastric mucosa, *Muntingia calabura*.

A significant increase in the world's soft drink consumption occured within the past two decades.(1) A Randomized controlled crossover trial study suggested that sugar-sweetened drinks consumption in a moderate amount for three weeks could elevate inflammation biomarker levels, the high sensitivity C-reactive protein, up to more than 60%.(2)

Chronic alcohol consumption has been known as to cause gastrointestinal diseases such as peptic ulcer, gastroesophageal reflux disease (GERD), liver and kidney diseases, psychological disorders, and even cancer.(3) These conditions resulted from the chronic process of alcohol metabolism within the body, causing much carcinogenic agents such as Reactive Oxygen Species (ROS) to build up within certain organs particularly the gastrointestinal organs. ROS destroys cell components, alter NADH to NAD+ ratio, leads to tissue injury, metabolic alterations, causing cancer, and drugs interaction.(4)

The use of medicinal plants extraction has drawn such interest within the discovery of better treatment for unhealthy diet-related diseases such as gastric ulcer. Natural products has become such trends and that the use of natural source for drugs such as medicinal plants extracts is likely to be more favorable.

One of the potential medicinal plants to alleviate gastric ulcer is *Muntingia calabura*, commonly known as *talok* or *kersen* (Java, Indonesia), *kerukup siam* (Malaysia), or Jamaican cherry. *Muntingia calabura* is a

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perennial plant which can be found easily as a roadside trees in tropical Asian countries such as Indonesia.(5) Previous studies suggested *Muntingia calabura* as a great source of flavonoids which act as potent antioxidant agents, with the highest content of flavonoids lay within its leaves.(6)(7) *Muntingia calabura* leaves extract exhibited significant pharmacological effects such as antiulcer, antiinflammatory, antinociceptive, antimicrobes, and anticancer.(7) Despite its known pharmacological properties, there is little we know about how *Muntingia calabura* leaves extract alleviates the morbidity caused chronic consumption of soft drink and alcoholic beverages.

MATERIALS AND METHODS

This study is a quasi experimental with post test only control group study consists of four groups of male wistar rats receiving treatments for a whole 30 days course of treatments. The alcoholic beverage used was whisky, and the soft drink used was soft drink with the brand name initial "CC".

Plant specimen and extract preparation

Muntingia calabura leaves were obtained from Faculty of Medicine Diponegoro University, Indonesia and identified by the biologist of Faculty of Science and Math Diponegoro University. The dried leaves was then ground using electrical grinder. One hundred grams of the finest ground *Muntingia calabura* leaves were soaked in 500 ml 95% ethanol for 72 hours. After 72 hours, the mixture were filtered using muslin cloth followed by filter paper (Whatman No.1) and distilled under low pressure with 40°C temperature in an Eyela rotary evaporator (Sigma-Aldrich, USA). The dry extract was then dissolved in CMC (0,5% w/v) to be given orally to the experimental rats with the dose of 500 mg/kgBW (5 ml/kgBW) *Muntingia calabura* ethanolic leaves extract (MCELE).(8)

Experimental animals and inductions of gastric mucosal damage by soft drink and alcoholic beverage

The study required healthy male wistar rats with body weight ranged within 200–300 grams, 8-12 weeks old which were obtained from Integrated Research and Testing Laboratory of Gajah Mada University, Indonesia. The rats were placed in a separated cage and were adapted for one week before receiving any treatments. The rats were given pellets and water ad libitum. The study was approved by Ethics Committee for Medical Research Faculty of Medicine Diponegoro University, Indonesia. The experimental rats were chosen and treated according to Institutional Animal Care and Use Committee Guidebook from National Institutes of Health and World Health Organization's General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine.

The gastric mucosal damage were induced by the administrations of 50 ml/day soft drink ad libitum and alcoholic beverages with ethanol percentage of 40% (whisky) as much as 1,8 ml/200gBW given with oral gavage. The dosage was calculated in aim to reach the gastric mucosal damage level wanted within 30 days of treatments. Twenty four male wistar rats divided into four groups respectively treated with 1,8 ml/200grBW 40%-alcoholic beverages (K1), 50 ml/day soft drink ad libitum (K2), pre-treated with 500 mg/kg MCELE one hour before oral administration of 40%-alcoholic drinks (P1) and pre-treated with 500 mg/kg MCELE one hour before oral administration of soft drink ad libitum (P2). The rats were sacrificed on day 31st

Histological evaluation of gastric mucosal damage

Gastric tissue samples were obtained and sent to anatomical pathology laboratorium of Kariadi Central Public Hospital in order to process them into preparations using microtechnique and HE staining. Histological evaluation on gastric mucosal lesions were determined by using modified scale of epithelial mucosa integrity *Barthel-Manja.*

Statistical analysis

The data collected was then assessed for its statistical significance of differences between groups using Kruskal-Wallis statistical test followed by Mann-Whitney test. A value of $p \leq 0,05$ was considered significant.

RESULTS

Descriptive analysis

In the present study, there were no macroscopic bleeding found on the gastric mucosa. However, some of the gastric samples from K1 group had irregular shape when distended, which was caused by the

thinning of the gastric mucosa on several areas, notably at fundus and corpus area. Gastric samples obtained from K1 and K2 groups both had macroscopic mucosal thinning, erosions, and reduced rugae when the gasters were incised open along the great curvature of the gaster. Otherwise, the gastric samples from P1 and P2 groups showed better mucosal condition compared to K1 and K2 groups, with the rugae still noticeable and mucosal thickness similar to normal gastric mucosa of a rat.

Microscopic examination showed a remarkable changes on gastric mucosal integrity; from no pathologic changes (normal mucosa) to epithelial desquamation, epithelial surface erosion (gap 1-10 epithelial cells/lesion), and epithelial ulceration (gap >10 epithelial cells/lesion).

In K1 group, microscopically the gastric mucosa had epithelial surface erosion up to 46,7% and ulceration up to 33.3% (Figure 1a.). The K2 group which were treated with only softdrink, showed microscopic appearance of gastric mucosa with almost no distinct features than K1 group (Figure 1b.). Both groups had 0,0% normal epithelial found.

Otherwise, P1 and P2 groups were dominated by epithelial desquamation and normal epithelial with no ulceration found. In P1 group, the epithelial desquamation was observed up to 66,7% (Figure 1c.) while in P2 group it was observed that there was 53,3% epithelial desquamation (Figure 1d.). Both groups showed minimal epithelial surface erosion with percentage of only 6,7%. The frequencies and percentages of the gastric histopathological observation after treatment is shown in Table 1.

Inferential analysis

Kruskal-Wallis test was performed between experimental groups and it showed a significant difference between them (p<0,001). A Post Hoc test of Kruskal-Wallis test, Mann-Whitney test was perfomed following the previous mentioned test. Mann-Whitney test for K1 vs P1 and K2 vs P2 both showed significant differences with p value of p<0,001. Meanwhile, K1 vs K2 showed no signficant difference with p value of p=0,293 or p>0,05.

DISCUSSION

The exposures of destructive agents such as ethanol, water restraint stress, or ischemia followed by reperfusion on gastric mucosa will lead to pathological alterations in a form of inflammation process, haemorrhagic erosion, and acute ulcer. The basis of these alterations disturbs the protective mechanisms and impairs the gastric mucosal defense. The previous studies suggested that there are involvements of the impaired gastric blood flow, mucous secretion, and the role of prostaglandin and NO generation in the pathomechanism of gastric mucosal lesions formation.(9) Gastric lesions by ethanol are commonly associated with ROS generation, whereby these lesions produce an imbalance between oxidant and antioxidant cellular processes. This is evidenced by increased levels of malondialdehyde, a marker of increased lipid peroxidation.(6) The deleterious effect by ethanol manifests through either via direct reactive metabolites generation or contributing to other mechanisms that finally support oxidative damage.(10)

The imbalance of aggressive factors and protective factors of gaster triggers acute inflammation reaction. Interleukin-1 beta (IL-ß) and tumor necrosis factor alpha (TNFα) are the major proinflammatory cytokines which hold important roles in creating acute inflammatory reaction followed by neutrophile infiltration within the gastric mucosa. Neutrophile produces radical anion superoxide (O_2^-) which is one of the ROS kinds, and reacts with cellular lipids, resulting in the formation of lipid peroxides which are then metabolized into malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), two of the metabolytes indicating the presence of mucosal injury by ROS. The concentrations of 4-HNE and MDA in intact mucosa measured at low levels, whereas in the 100% ethanol-exposed mucosa they were found to be twice as high. The damage on the gastric mucosal integrity induced by ethanol may be caused by decrease in gastric blood flow, increase in inflammatory changes indicated at the increased level of IL-ß and TNFα and the ROS production as well as the endogenous antioxidant activity attenuation within the cells.(9)

Chronic consumption of soft drink are related to increased oxidative stress processes.(11) The high content of fructose and sucrose within soft drink triggers malabsorption and increases any inflammation event in the gaster which are associated with atrophic gastritis events and

paracellular leakage of gastric mucosa.(12)(13)Constant irritation from the acidity, gas content, high sucrose and fructose content, and the presence of bysphenol A (BPA) in a significant level within soft drink induce alterations of gastric mucosal integrity as a result of continuous oxidative stress process and decreased activity of endogenous cellular antioxidant which is caused by a decrease in the levels of antioxidant proteins.(11)(14) One of the previous study suggested that gastric cells exposed to nonylpolyphenols, a form of of BPA, in a certain dose with maximal cytotoxic dose of 10^{-7} M within 48 hours would have its cell cycle and apoptosis rate altered.(15)

Nitric oxide (NO) acts as both intercellular and intracellular messenger. It acts in two different ways in stomach; NO induces the activation of defensive factors of gaster, but when overproduced it causes inflammation process including ROS and NO generation which caused gastric ulcer, chronic gastritis, bacterial gastroenteritis, and other gastrointestinal diseases in the end.(16)

Present study showed an insignificant histopathological difference between K1 and K2 groups (p=0,293), which could be interpreted as the exposure of soft drink for 30 days with a dose of 36-39 ml/day in wistar rats generated deleterious effect on gastric mucosa as potent as 40%-ethanolic beverages exposure with the equal duration. The volume range of soft drink required to induce desquamation–ulceration lesions in human when consumed everyday for at least 30 days is 256–275 ml. Destructive effects of both soft drink and alcoholic beverages come from the generation of free radicals and ROS when the gastric epithelial cells are exposed to both drinks. The high level of ROS oxidizes the lipid within cell membranes directly and induces peroxidation, prolonged the degeneration on gastric cells and resulted in producing desquamation, erosion, evenmore ulceration on the gastric mucosa.(11)

Microscopic examinations on the wistar rat gaster which had been given MCELE 500 mg/kgBW prior to administration of 40%-alcoholic beverages 1,8 ml/200grBW suggested that there is gastroprotective effect exerted by MCELE, protecting the gastric mucosal integrity and help strengthen the mucosa. It is ascertained from the result of comparison between K1 group to P1 group with resulting p value of <0,001 and the proportions of normal gastric mucosal integrity in P1 group was much higher (26,7%) than in K1 group (0,0%). In K2 and P2 groups comparison, there was significant difference with resulting p value of <0,001 and again, the higher proportion of normal gastric mucosal integrity was found in P2 group compared to K2 group. The ulcerated gastric mucosa was found to be higher in K1 group than in K2 group (33,3% and 23,3% respectively) and none was found in P groups.

Various studies examining the active compounds within *Muntingia calabura* conducted from 1991 to 2013 had identified 86 bioactive compounds isolated from various parts of the plant, including leaves, barks, flowers, and fruits in various forms of extractions. The bioactive compounds mainly found were flavonoids and its derivatives, chalcones, other phytosterols, and some organic acids such as syringic acid and vanilic acid.(7) A study of phytochemical analysis of *Muntingia calabura* leaves conducted in Indonesia in 2014 showed that the highest concentration of flavonoid obtained from the extraction process using polar solvents such as ethanol, methanol, and water. The common bioactive compounds identified in a large quantities were epigallocatechin gallate (EGCG) and genistein, both of those constituents are part component of catechin, one of the most powerful antioxidant mainly found in tea that are thought to provide several health benefits.(17) The peak plasma concentrations of EGCG are reached in 1-2 h in healthy subjects with one oral dose in the morning after an overnight fasting period then diminish gradually to undetectable levels in 24 hours.(15)(18)

Antioxidant activity of flavonoids, including those residing within *Muntingia calabura* leaves, comes from its capacity as free radical scavenging, breaks the chain of free radical formation reactions, decreases the amount of peroxides, and activating various endogenous antioxidant proteins within its interaction on oxidative stress signalling pathway.(19) *Muntingia calabura* leaves extract in various fraction solution using different kinds of solvent at a concentration of 100mg/ml showed NO-inhibiting activity in macrophage-induced inflammation and help maintaining cell viability.(6) Zakaria et al (2007) conducted a study on antioxidant activity of aquoeus extract of *Muntingia calabura* leaves ($AEMC_L$) using DPPH free radical-and superoxide anion radical scavenging assays method, and it showed that it had 94,80 ± 1,14 and 83,70 ± 2,05% measured antioxidant

capacity.(20) Otherwise, the antiinflammatory activity of *Muntingia calabura* leaves had also been studied. $AEMC_L$ of 10% and 50% concentration (27 and 135 mg/kg consecutively) have much higher antiinflammatory activity observed at 3 and 4 hours intervals after administration compared to acetylsalycilate acid 100 mg/kg as a reference drug.(21)

Flavonoid compound consists of one or more aromatic ring which have one or more hydroxil groups, giving flavonoid its ability to neutralize free radicals by converting them into resonance-stabilized phenoxyl radicals.(1) Other than that, flavonoids found in *Muntingia calabura* leaves increase mucus production, which has been known as gastric mucosa defense mechanism against corrosive and oxidative agents.(22) Previous study using MCELE of 250 mg/kg and 500 mg/kg compared to omeprazole 20 mg/kg as a reference drug showed that MCELE has significant and dose-dependet antiulcer activity when compared to omeprazole. It is also suggested that MCELE reduces the acidity of gastric juice and enhances mucous production within ethanol-induced gastric ulcers in rats.(8) The other study conducted in 2013 used *Muntingia calabura* methanolic leaves extract (MCMLE) and ranitidine as reference drug on ethanol-induced gastric ulcer model suggested that MCMLE has significant and in dose-dependent manner antiulcer activity. Histological examination showed that MCMLE has a potential to reverse toxic effect of ethanol and helps regenerate mucosal structure to its normal condition as shown in ranitidine administration. These abilities may have related to mechanism involving NO modulation and endogenous sulfhydril compounds. The ability of MCMLE fractions to prevent ethanol-induced gastric ulcer suggests the involvement of local and non-specific mechanism called adaptive cytoprotection.(6)

Finally, we suggest that MCELE has a potential to be used as an alternative source of medicine to treat degenerative disease, especially gastric-related disease. Further studies are required to identify the exact bioactive compounds that may be responsible for the antiulcer properties of *Muntingia calabura*.

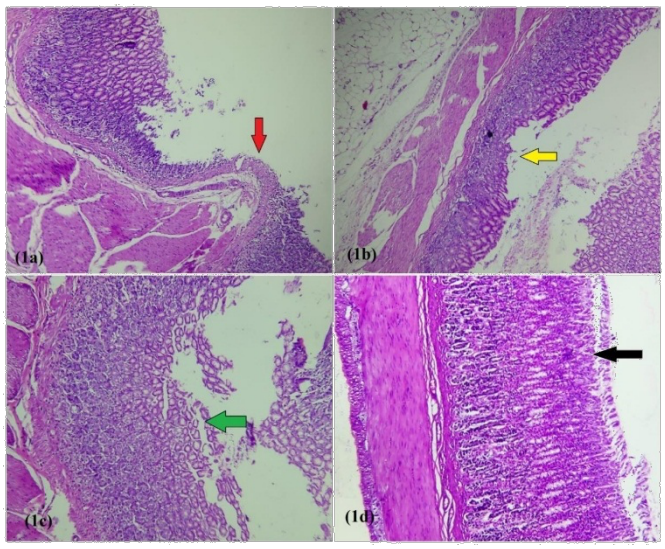

Figure 1. Histological examination of gastric mucosal integrity. The samples were examined using light microscope with the aforementioned magnifications. (1a.) HE staining (×100). The ulceration was shown (red arrow) and found most abundant in K1 goup. (1b.) HE staining (×100). The epithelial surface erosion was shown (yellow arrow), K2 group was dominated by this kind of lesion. (1c.) HE staining (×100). Black arrow points to the epithelial desquamation, which was dominating in P1 group, indicating gastroprotective effect exerted by MCELE given to this group. (1d.) HE staining (×40). P2 group showed the most abundant intact mucosa (red arrow) as indication to the gastroprotective effect of MCELE given to this group.

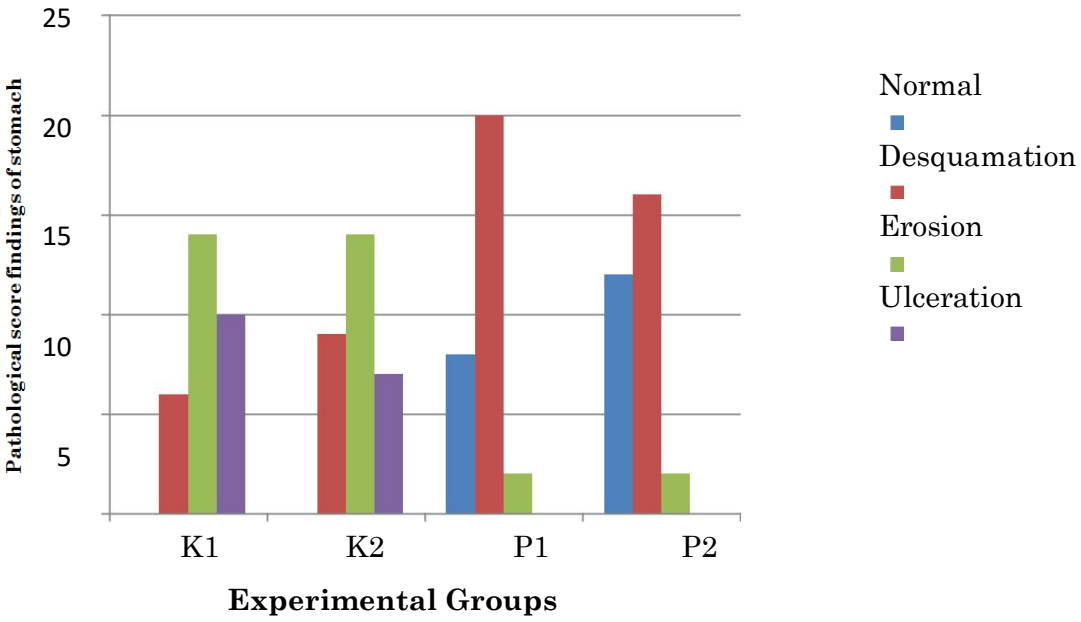

Figure 2. Result of pathological score findings of the experimental groups.

	Normal	Epithelial desquamation	Epithelial surface erosion	Ulceration	Total
K1	0.0% (0)	20.0% (6)	46.7% (14)	33.3% (10)	100% (30)
K2	0.0% (0)	30.0% (9)	46.7% (14)	23.3% (7)	100% (30)
P1	26.7% (8)	66.7% (20)	6.7% (2)	0.0% (0)	100% (30)
P2	40.0% (12)	53.3% (16)	6.7% (2)	0.0% (0)	100% (30)
Total	**16.7% (20)**	**42.5% (51)**	**26.7% (32)**	**14.2% (17)**	**100% (120)**

Table 1. Frequencies and percentages of the histopathologic examination on gastric experimented rats.

	***p* value**			
Group	**K1**	**K2**	**P1**	**P2**
K1	-	0.293	0.000*	-
K2	0.293	-	-	0.000*
P1	0.000*	-	-	0.338

Table 2. Mann-Whitney non-parametric test of the result on gastric mucosal integrity scoring and examination

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